The Adenosine Triphosphatase-Inhibitor Content of Bovine Heart Submitochondrial Particles

INFLUENCE OF THE INHIBITOR ON ADENOSINE TRIPHOSPHATE- DEPENDENT REACTIONS

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1. The activity of the ATPase (adenosine triphosphatase) of phosphorylating particles prepared by sonication of bovine heart mitochondria in the presence of $MgCl_2$ and ATP is influenced by the isolation method for the mitochondria used in the preparation of particles. Type-I particles, made from mitochondria isolated in a medium lacking succinate, have a lower ATPase activity than do Type-II particles, which are prepared from mitochondria isolated in a medium containing succinate. 2. Centrifugation under appropriate energized conditions increases the ATPase activity of Type-I particles almost to that of the Type-II particles. The ATPase activity of Type-II particles was only slightly stimulated by this procedure. These data are interpreted as indicating a higher content of the ATPase-inhibitor protein in the Type-I particles. 3. A comparison was made of the ATP-driven enhancement of 8-anilino-naphthalene-1-sulphonate fluorescence and the exchange of the endogenous tightly bound nucleotides of the ATPase in Type-I and Type-II particles. The effect of exogenous inhibitor protein on both these reactions was also studied. 4. The time-scale on which the inhibitor protein can exchange between ATPase molecules is discussed.

A naturally occurring ATPase* inhibitor can be isolated from bovine heart mitochondria (Pullman & Monroy, 1963). This is a soluble trypsin-sensitive protein of mol.wt. 10 500 (Horstman & Racker, 1970; Senior & Brooks, 1971) that strongly inhibits the ATPase activity both of some preparations of submitochondrial particles and of soluble isolated mitochondrial ATPase (Asami *et al.*, 1970; Horstman & Racker, 1970; van de Stadt *et al.*, 1973). By contrast, oxidative phosphorylation in submitochondrial particles is not inhibited (Pullman & Monroy, 1963; Asami *et al.*, 1970). The effect of the ATPase inhibitor on ATP-dependent reactions in submitochondrial particles is less clear. Asami *et al.* (1970) showed that a variety of ATP-driven reactions, such as energy-dependent reduction of NAD^+ by succinate and enhancement of 8-anilino-naphthalene-1-sulphonate fluorescence, were inhibited when the ATPase inhibitor was added to EDTA particles. However,

the ATPase inhibitor had little effect on either ATPase or ATP-dependent reactions of Mg-ATP particles (Ernster *et al.*, 1973). Lang & Racker (1974) have also reported that the ATPase inhibitor does not inhibit the ATP-dependent reduction of NAD^+ by succinate in submitochondrial particles.

It is thought that Mg-ATP particles are rich in ATPase inhibitor (Ernster *et al.*, 1973). The relative insensitivity of ATP-utilizing reactions in these particles would then be a simple consequence of added inhibitor only slightly increasing the proportion of ATPase molecules that are inhibited. Clarification of this point would be best achieved by studying the effect of the ATPase inhibitor on inhibitor-depleted Mg-ATP particles. Ernster *et al.* (1973) found that treatments that removed the ATPase inhibitor from Mg-ATP particles uncoupled the particles, but van de Stadt *et al.* (1973) showed that centrifugation of Mg-ATP particles that were catalysing oxidative phosphorylation produced inhibitor-depleted particles that were still well coupled.

In the present paper we show that the ATPase-inhibitor content of Mg-ATP particles varies according to the method used for isolating the mitochondria from which the particles are made, and that inhibitor-depleted Mg-ATP particles can be prepared directly from appropriate mitochondria. A further purpose of the present study was to investigate the effects of the ATPase-inhibitor

* Abbreviations: ATPase, adenosine triphosphatase; Mg-ATP particles, phosphorylating particles prepared by sonication of bovine heart mitochondria in the presence of 15 mM- $MgCl_2$ and 1 mM-ATP; Type-I particles, Mg-ATP particles prepared from mitochondria that were isolated in the absence of succinate; Type-II particles, Mg-ATP particles prepared from mitochondria that were isolated in the presence of succinate; EDTA particles, non-phosphorylating particles obtained by sonication of mitochondria at alkaline pH in the presence of EDTA.

protein on the energy-linked enhancement of 8-anilino-naphthalene-1-sulphonate fluorescence and the exchange of the tightly bound adenine nucleotides of the mitochondrial ATPase in submitochondrial particles (Harris *et al.*, 1976). We also discuss whether the inhibitor can be regarded as acting 'irreversibly'.

Materials and Methods

Heavy-layer bovine heart mitochondria were prepared either by the method of Low & Vallin (1963) (except that a Waring Blendor was used instead of an Ultra-Turrax homogenizer), in which succinate is absent from the isolation medium in the final stages of the preparation, or by procedure 3 of Smith (1967), which uses solutions containing succinate throughout. The mitochondria made in the absence of succinate were stored at -20°C in 0.25 M-sucrose, and were used to prepare Type-I Mg-ATP submitochondrial particles. Type-II particles were prepared from the mitochondria that were isolated in a succinate medium and that were stored at -20°C in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.8)/1 mM-succinate/0.2 mM-EDTA. The Mg-ATP particles were prepared by the method of Low & Vallin (1963). ATPase-inhibitor protein was prepared by the method of Nelson *et al.* (1972) and combined with submitochondrial particles as described by van de Stadt *et al.* (1973). Changes in 8-anilino-naphthalene-1-sulphonate fluorescence and ATPase activity were measured as described previously (Ferguson *et al.*, 1976a,b) unless otherwise indicated, and exchange of tightly bound nucleotides of the mitochondrial ATPase was measured by the method of Harris *et al.* (1977). Protein was determined by the biuret method as modified by Cleland & Slater (1953). Chemicals were obtained from the sources specified previously (Ferguson *et al.*, 1976a), and [^3H]ATP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Type-I and Type-II Mg-ATP submitochondrial particles were prepared in parallel from mitochondria, and their ATPase activities compared. Table 1 shows that the Type-II particles had a considerable higher ATPase activity than Type-I particles. Data given in Table 1 for Tris/acetate buffer can be compared with measurements of ATPase activities in submitochondrial particles determined by others (e.g. Ernster *et al.*, 1973); activities in the mannitol/sucrose/Tris buffer are given, as these are the conditions used for 8-anilino-naphthalene-1-sulphonate fluorescence measurements (Ferguson *et al.*, 1976a).

To determine if the ATPase activities of Type-I and Type-II particles were a reflection of the relative

Table 1. ATPase activities of Type-I and Type-II submitochondrial particles, showing changes in ATPase activity produced by depleting the particles of ATPase inhibitor

The reaction was started by addition of particles to the appropriate assay medium containing 2 mM-ATP and 2 mM-MgCl₂, and the rate of reaction was measured during the first 2 min of the reaction.

Assay medium	ATPase activities ($\mu\text{mol}/\text{min per mg}$)			
	Particle ...	Type I	Type I*	Type II Type II*
Mannitol/sucrose/Tris buffer, pH 7.4, 25°C		0.16	0.72	0.84 1.26
50 mM-Tris/acetate, pH 7.5, 30°C		0.42	1.85	2.2 3.6

* Inhibitor-depleted particles.

Fig. 1. Slow dissociation of ATPase inhibitor at high ionic strength

Type-I or Type-II particles (4 mg/ml) were incubated in 60 mM-triethanolamine hydrochloride/60 mM-KCl/30 mM-sucrose/4 mM-MgCl₂/1.5 mM-EDTA, pH 7.5, at either 32°C or 42°C as shown. ATPase activity was measured at pH 7.5, 32°C, in 30 mM-KCl/5 mM-Tris/acetate/3 mM-MgCl₂/1 mM-EDTA/2 mM-KCN/1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide, by coupling ATP hydrolysis to NADH oxidation as described by Ernster *et al.* (1976). ATPase rates were linear with respect to time for at least 4 min. The ATPase activities are slightly higher than those measured in 50 mM-Tris/acetate (Table 1). This difference is attributed to the higher temperature of assay, the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide as uncoupler, which slightly stimulated the activity, and the different ionic strength of the assay medium. ○, Type I, 32°C; ●, Type I, 42°C; □, Type II, 32°C; ■, Type II, 42°C.

contents of ATPase inhibitor in the particles, both types of particles were depleted of the inhibitor by centrifugation under the phosphorylating conditions specified by van de Stadt *et al.* (1973). This procedure considerably stimulated the ATPase activity of Type-I particles, but had proportionately much less effect on the activity of Type-II particles (Table 1). It may be concluded that Type-I particles contain much more ATPase inhibitor than do Type-II particles. Also, Type-II particles were not produced from mitochondria that were isolated in the absence of succinate, even when succinate was added to the mitochondria immediately before sonication.

van de Stadt & van Dam (1974) reported that the ATPase activity of Mg-ATP particles was stimulated by high ionic strength (50mM-KCl), which induced a partial dissociation of the ATPase inhibitor. Fig. 1 shows that activation of ATPase in Type-I particles by high ionic strength is very slow, in line with the observations of Pullman & Monroy (1963), who showed that interactions between isolated mitochondrial ATPase and the inhibitor were also very slow. Type-II particles were hardly stimulated by incubation at high ionic strength, which again indicates that these particles are depleted of inhibitor. Fig. 1 also shows that high ionic strength alone does not prevent the suppression of ATPase by the inhibitor without a long preincubation, and this should be taken into account when measuring ATP-dependent reactions and ATPase in submitochondrial particles.

Influence of the ATPase inhibitor on the ATP-driven 8-anilidonaphthalene-1-sulphonate fluorescence enhancement

The fluorescence of 8-anilidonaphthalene-1-sulphonate is well established as a probe for detecting energization of submitochondrial particles (Azzi *et al.*, 1969). 8-Anilidonaphthalene-1-sulphonate fluorescence has been used to monitor the extent of

ATP-supported energization of Mg-ATP submitochondrial particles as the ATPase activity was progressively decreased by irreversible (covalent) inhibitors of the ATPase (Ferguson *et al.*, 1976a,b). It was therefore expected that the ATP-dependent enhancement of 8-anilidonaphthalene-1-sulphonate fluorescence would reflect the ATPase-inhibitor protein content of the Mg-ATP particles.

Energization of Type-II particles, by either succinate or ATP, produced very similar enhancements of 8-anilidonaphthalene-1-sulphonate fluorescence (Table 2). A smaller increase in 8-anilidonaphthalene-1-sulphonate fluorescence was observed when Type-I particles were energized with ATP, but succinate produced a fluorescence enhancement comparable with that seen with Type-II particles (Table 2). The ATP- and succinate-supported 8-anilidonaphthalene-1-sulphonate fluorescence enhancements were equalized after Type-I particles had been depleted of ATPase inhibitor by the procedure of van de Stadt *et al.* (1973) (Table 2). This procedure had no effect on either the ATP-dependent 8-anilidonaphthalene-1-sulphonate fluorescence enhancement in Type-II particles or the succinate-supported fluorescence enhancement in either kind of particle (Table 2). The 8-anilidonaphthalene-1-sulphonate fluorescence thus senses the increased ATP-dependent energization of the Type-I particles after removal of ATPase inhibitor. Similar results have been obtained by Ernster *et al.* (1976).

Energy-linked changes in 8-anilidonaphthalene-1-sulphonate fluorescence are characterized not only by the extent of fluorescence enhancement, but also by a pseudo-first-order rate constant that describes the rate of fluorescence change (Ferguson *et al.*, 1976a). Type-I particles consistently gave slower rates of enhancement than Type-II particles. Typically, under the reaction conditions specified in Table 2, the rate constant for Type-I particles was 0.06s⁻¹ and that for Type-II particles was 0.12s⁻¹. This difference in rate of response was unexpected,

Table 2. Comparison of ATP- and succinate-driven 8-anilidonaphthalene-1-sulphonate fluorescence enhancement in Type-I and Type-II submitochondrial particles

Fluorescence enhancement is defined as:

$$\frac{\text{8-anilidonaphthalene-1-sulphonate fluorescence with energized particles}}{\text{8-anilidonaphthalene-1-sulphonate fluorescence with resting particles}}$$

In each case the fluorescence changes were measured in a medium containing 2ml of 20mM-Tris/HCl buffer, pH7.4, 225mM-mannitol and 75mM-sucrose, 5μM-8-anilidonaphthalene-1-sulphonate and approx. 0.15mg of particles at 25°C; 2mM-MgCl₂ and 2mM-ATP or 5mM-succinate were added as appropriate.

Energizing substrate	Particle	Fluorescence enhancement			
		Type I	Type I*	Type II	Type II*
ATP		2.5	4.5	4.2	4.0
Succinate		4.0	4.0	4.1	4.2

* Indicates inhibitor-depleted particles.

Fig. 2. Double-reciprocal relationship between energy-linked 8-anilidonaphthalene-1-sulphonate fluorescence increase and ATPase activity of submitochondrial particles which have been titrated with inhibitor protein

Inhibitor-depleted Type-I particles were combined with different amounts of inhibitor protein and the fluorescence changes and ATPase activity measured.

as it has been shown that the rate constant for the ATP-driven 8-anilidonaphthalene-1-sulphonate is unchanged as the ATPase activity of submitochondrial particles is decreased by irreversible inhibitors (Ferguson *et al.*, 1976a).

The variation in the rate constant and extent of 8-anilidonaphthalene-1-sulphonate fluorescence enhancement as a function of the ATPase-inhibitor protein concentration was investigated further by titration of Type-II particles with inhibitor. A decrease in ATPase activity caused a parallel decrease in the extent of energy-linked fluorescence enhancement. Fig. 2 shows that a double-reciprocal plot of energy-linked fluorescence increase against ATPase activity gave a straight line, just as was observed when *NN*-dicyclohexylcarbodi-imide and 4-chloro-7-nitro-benzofurazan were used as inhibitors of ATPase (Ferguson *et al.*, 1976a). It may be concluded that the energy level of the particles does follow the ATPase activity.

Brief justification for plotting reciprocal fluorescence against reciprocal ATPase activity is apposite. When the 8-anilidonaphthalene-1-sulphonate concentration is low compared with the concentration of binding sites, a double-reciprocal plot of fluorescence against submitochondrial-particle concentration will be linear, irrespective of the number of types of binding site (Gains & Dawson, 1975). Fig. 2 is plotted on the assumption that decreasing ATPase activity is formally equivalent to decreasing the concentration of particles. The energy-linked increase in fluorescence is plotted, rather than the total fluorescence, because the contribution to the fluorescence of the 8-anilidonaphthalene-1-sulphonate that is

Table 3. Variation of the pseudo-first-order rate constant for 8-anilidonaphthalene-1-sulphonate fluorescence changes as ATPase activity is decreased by titration with inhibitor protein

In Expt. A, inhibitor-depleted Type-I submitochondrial particles (0.6mg/ml) were combined with different amounts of ATPase inhibitor as described by van de Stadt *et al.* (1973). In Expt. B, Type-II submitochondrial particles (2.1mg/ml) were combined with the amounts of inhibitor shown. The effect of inhibition of ATPase by dicyclohexylcarbodi-imide on the rate constant for fluorescence enhancement was checked in parallel with Expt. B. Up to 86% inhibition of ATPase activity by dicyclohexylcarbodi-imide did not change the rate constant for fluorescence enhancement. *k* is as defined previously (Ferguson *et al.*, 1976a).

	Inhibitor protein added (μg/ml)	Inhibition of ATPase (%)	<i>k</i> (s ⁻¹)
Expt. A	0	0	0.096
	1.25	20	0.092
	2.5	43	0.077
	5.0	63	0.070
	10	73	0.080
Expt. B	0	0	0.110
	1.1	38	0.070
	4.3	52	0.067

bound to the particles in the absence of energization will remain constant as the ATPase activity is decreased. The contribution of the non-energy-linked 8-anilidonaphthalene-1-sulphonate fluorescence is unaffected by energization, and so it is legitimate to consider only the energy-dependent increase in fluorescence in Fig. 2. Examination of the kinetics of 8-anilidonaphthalene-1-sulphonate binding to energized submitochondrial particles clearly shows a fast phase corresponding to the non-energy-linked binding sites, and a slower component corresponding to the energy-linked binding (Ferguson *et al.*, 1976a).

Table 3 shows that, as the ATPase activity was progressively inhibited, the rate constant for the ATP-dependent 8-anilidonaphthalene-1-sulphonate fluorescence enhancement also decreased. Two experiments are shown, one with inhibitor-depleted Type-I particles, the other with Type-II particles. In agreement with previous results (Ferguson *et al.*, 1976a), titration of the Type-II particles with dicyclohexylcarbodi-imide did not change the rate constant for fluorescence enhancement, but progressively decreased the extent of enhancement.

Influence of the ATPase inhibitor on the energy-linked exchange of the endogenous bound nucleotides of the mitochondrial ATPase

The mitochondrial ATPase contains very tightly bound ATP and ADP (Harris *et al.*, 1973), and in

Table 4. Exchange of bound nucleotides of the ATPase in Type-I and Type-II submitochondrial particles

Exchange was measured by incubating the particles (2mg/ml) at 30°C in 3ml of a solution containing 200 μ mol of triethanolamine hydrochloride, 120 μ mol of KCl, 120 μ mol of sucrose, 9 μ mol of $MgCl_2$, 3 μ mol of EDTA, 30 μ mol of phosphoenolpyruvate, 30 units (μ mol/min) of pyruvate kinase (NH_3 -free) and 0.5 μ mol of [3H]ATP (3000 c.p.m./nmol) at pH 7.5. After 5 min the suspension was diluted with cold sucrose solution (250 mM) containing $MgCl_2$ (10 mM) and the particles were collected by centrifugation (100 000 g for 20 min) at 0°C. The particles were then washed by repeated centrifugation until no radioactivity appeared in the supernatant (four washes were usually sufficient) and the radioactivity in the particles was counted as described by Harris *et al.* (1976). The data given here have been corrected for a non-energy-linked exchange of 0.33 mol/mol of ATPase, which was not inhibited by either oligomycin or uncouplers, and was independent of the type of particle preparation. The data are calculated assuming that there are 0.42 nmol of ATPase/mg of particle protein (Harris *et al.*, 1976).

	[3H]Adenine nucleotide exchanged (mol/mol of ATPase)
Type-I particles	0.6
Type-I particles, inhibitor-depleted	1.1
Type-II particles	1.2

submitochondrial particles the sole pool of tightly bound ATP and ADP is associated with the ATPase (Harris *et al.*, 1976). These nucleotides exchange with added ATP if the submitochondrial particles are energized by ATP or substrates of the respiratory chain (Harris *et al.*, 1976). Exchange of the bound nucleotides is inhibited by uncouplers.

Type-II particles showed a higher ATP-dependent exchange of bound nucleotides than did Type-I particles (Table 4). After removal of inhibitor protein, Type-I particles had an exchange comparable with that by Type-II particles. It may be concluded that the ATPase inhibitor suppresses the exchange reaction, as do some other inhibitors of the ATPase (Harris *et al.*, 1976). Fig. 3 shows that the exchange of bound nucleotides decreases as the ATPase activity of Type-II particles is titrated with inhibitor protein, and that the decrease in exchange parallels the decrease in the extent of the 8-anilino-naphthalene-1-sulphonate fluorescence enhancement. These data are again consistent with the view that Type-II particles contain less inhibitor protein than do Type-I particles.

Discussion

Our results show that the ATPase-inhibitor content of Mg-ATP submitochondrial particles depends

Fig. 3. Dependence of exchange of bound nucleotides and 8-anilino-naphthalene-1-sulphonate fluorescence enhancement on ATPase activity in Type-II particles

Different amounts of ATPase inhibitor were combined with particles, and samples taken for measurements of fluorescence and ATPase activity, which was assayed in 20 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid titrated to pH 7.1 with Tris/250 mM-sucrose/3 mM- $MgCl_2$ /1 mM-EDTA, as described by Harris *et al.* (1976). This is a low-ionic-strength buffer, so as to avoid dissociation of the inhibitor from the particles. The remaining particles were collected by centrifugation and resuspended in the same buffer. The bound nucleotide exchange was measured as in Table 4. Δ , Fluorescence enhancement (%); $\bullet$, energized exchange of bound nucleotides (%).

on the isolation method for the mitochondria from which the particles are prepared. The presence of succinate throughout the preparation of mitochondria produces, on sonication, particles deficient in the ATPase-inhibitor protein (Type II). Particles derived from mitochondria isolated without succinate contain much more inhibitor protein (Type I), but these particles can be converted into Type-II particles by removal of inhibitor by centrifugation under appropriate energized conditions. The reason for the difference in inhibitor content of the two preparations is uncertain, especially as the presence of succinate during sonication of mitochondria isolated in the absence of succinate produced Type-I and not Type-II particles. Possibly, prolonged incubation of mitochondria with succinate, even at 0°C, results in some dissociation of inhibitor, which is degraded by proteinases in the mitochondrial preparation.

The present paper thus provides direct methods for preparing submitochondrial particles that are rich or deficient in the ATPase-inhibitor protein. By using

Type-II particles it is shown that the ATPase inhibitor suppresses both the ATP-driven enhancement of 8-anilidonaphthalene-1-sulphonate fluorescence and the ATP-dependent exchange of the bound nucleotides of the ATPase. Moreover, the activity of the ATP-driven reduction of NAD^+ by succinate was stimulated twofold (from 20 to 40 nmol of NAD^+ reduced/min per mg of protein) in Type-I particles after centrifugation of the particles under energized conditions to remove the inhibitor. All these results clearly show that the ATPase inhibitor does affect ATP-dependent reactions in Mg-ATP particles, and are in harmony with results from Ernster and co-workers, who showed that the ATPase inhibitor abolishes a wide range of ATP-dependent reactions in both EDTA submitochondrial particles (Asami *et al.*, 1970) and in what we term inhibitor-depleted Type-I particles (Ernster *et al.*, 1976). The report of Lang & Racker (1974) that the ATPase inhibitor did not inhibit the ATP-driven reduction of NAD^+ by succinate in submitochondrial particles, which should have been relatively deficient in inhibitor, is hard to reconcile with the present data. Possibly the particles used by Lang & Racker (1974) were in fact rich in inhibitor, so that exogenous inhibitor was added over an insensitive part of its titration curve, or they may have used an inadequate pre-incubation of the particles with the inhibitor in the presence of MgCl_2 and ATP. It should be noted that, since the rate of the ATP-dependent reactions varies with the type of particles used, it is meaningless to use the specific activity of an ATP-driven reaction as an index of the integrity of coupling of particles unless the inhibitor content is specified. This is true even at high salt concentrations (Fig. 1).

In a previous study on the effect of covalent inhibitors on the ATP-dependent 8-anilidonaphthalene-1-sulphonate fluorescence enhancement (Ferguson *et al.*, 1976a), Type-II particles were used. It was not originally realized that these particles were unusually deficient in ATPase inhibitor, but it is now apparent that, as was assumed, the titrations with covalent inhibitors were made over a wide range of total ATPase activity.

It is often assumed that the ATPase inhibitor acts virtually 'irreversibly' (cf. Ernster *et al.*, 1976), although this has never been unequivocally demonstrated. Any binding process is inherently reversible; the interaction of the inhibitor with the ATPase can only be 'irreversible' during a measurement time that is short relative to the rate of inhibitor dissociation from the ATPase.

Our data do not permit a clear definition of the time-scale on which the ATPase inhibitor may be classified as 'reversible' or 'irreversible'. The inhibitor decreases the extent of, and the rate constant for, the enhancement of 8-anilidonaphthalene-1-sulphonate fluorescence. Other covalent (irreversible)

inhibitors of the ATPase have been shown to decrease the extent of fluorescence enhancement, but to leave the rate constant unaltered (Ferguson *et al.*, 1976a). By this criterion the ATPase inhibitor does not act as an irreversible inhibitor on the time-scale of the fluorescence experiments, which have a half-time of approx. 10 s and are completed within 40 s. This may suggest that the inhibitor can exchange between ATPases on this time-scale. Alternatively the inhibitor may exert a secondary effect on the rate of fluorescence response, and in this respect it may be significant that the changes in rate constant are small relative to the decrease in ATPase activity (Table 3), whereas halving the nucleoside triphosphatase activity by substituting ITP for ATP also halved the rate constant for fluorescence enhancement (Ferguson *et al.*, 1976a).

The ATPase inhibitor inhibits the bound-nucleotide exchange, which is measured over a 5 min period. This would suggest that the inhibitor acts irreversibly on the long time-scale of the exchange experiment, as only one turnover per ATPase molecule is needed for an exchange to occur; any subsequent turnovers will not alter the specific activity of the bound nucleotides. Thus if the inhibitor could transfer between ATPases within 5 min, no inhibition of exchange would be expected. Aurovertin does not decrease the exchange even when present at sufficient concentration to inhibit the ATPase by over 80% (Harris *et al.*, 1976). This is the behaviour expected from an inhibitor that is reversible on the time-scale of the exchange experiment. Fig. 3 shows that the inhibition of exchange lags behind the inhibition of ATPase. The explanation for this behaviour is not clear, but it could arise from a transfer of a limited proportion of the inhibitor between ATPases during the experiment.

The combination of the fluorescence and bound-nucleotide-exchange experiments does not provide an estimate of the rate of transfer of ATPase inhibitor between ATPase molecules, which cannot easily be determined directly. From Fig. 1 it may be concluded that the release of inhibitor from submitochondrial particles is slow (half-time approx. 100 min at 42°C) in the absence of MgCl_2 and ATP. However, the fluorescence and nucleotide-exchange experiments are done in the presence of MgCl_2 and ATP, and it is therefore interesting to estimate the association and dissociation rate constants for the interaction of inhibitor and ATPase under these conditions. A second-order plot for the association reaction, using data taken from Fig. 1A in the paper of van de Stadt *et al.* (1973), gives a second-order rate constant of $2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. In these calculations the initial concentration of inhibitor was taken as 76 nM, assuming a mol.wt. of 10 500, and initial ATPase concentration was estimated at 41.5 nM,

assuming a mol.wt. of 360000, and that about 10% of the protein in submitochondrial particles is ATPase (Ferguson *et al.*, 1976b). It was further assumed that ATPase activity is inhibited by the binding of one molecule of inhibitor to an ATPase molecule. It is noteworthy that similar values have been obtained for other protein-protein association reactions that are probably diffusion-controlled (Vincent & Lazdunski, 1972).

Satre *et al.* (1975) measured a dissociation constant of $1 \times 10^{-7} \text{M}$ for the interaction between yeast mitochondrial ATPase and its inhibitor protein. Taking this dissociation constant as valid for bovine heart submitochondrial-particle ATPase, together with the association rate constant, a first-order rate constant for the dissociation of the ATPase-inhibitor complex may be estimated as $2 \times 10^{-2} \text{s}^{-1}$. Thus if this estimate is valid, the inhibitor cannot, in the presence of MgCl_2 and ATP, be regarded as being in rapid equilibrium between the ATPase and solution, but in measurements made in periods longer than 30s the reversibility might well become significant.

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